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The bioactivity of bacterium and fungi living associate with the sponge *Reniera* sp. against multidrug-resistant *Staphylococcus aureus* and *Escherichia coli*

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Abstract. *Trianto A, Nirwani, Susanti O, Maesaroh D, Radjasa OK. 2019. The bioactivity of bacterium and fungi living associate with the sponge* Reniera *sp. against multidrug-resistant* Staphylococcus aureus *and* Escherichia coli. *Biodiversitas 20: 2302-2307.* The study aimed to isolate and identify the sponge-associated microorganisms producing the antibacterial substances. The sponge *Reniera* sp. was collected by hand during skin diving in Karimunjawa Islands, Indonesia. The microbial symbionts were isolated with the dilution method and screened with the overlay method against the MDR *S. aureus* and *E. coli*. The bacterium was cultured in Zobell medium, while the fungi were cultured in malt extract broth (MEB) medium. The isolates were identified based on the molecular method. A total of 46 bacteria and 43 fungi were isolated, which 7 bacteria and 20 fungi exhibited antibacterial activity against the MDR *E. coli* and *S. aureus* strains. The molecular identification revealed that the active isolates close to *Pseudoalteromonas maricaloris* (99%), *Aspergillus nomius* (96%), *Eurotium rubrum* (99%), and *Penicillium* sp. (100%). Fractionation of K.J.16.U extract gave a fraction that active to the *S. aureus* and *E. coli* strains at concentration 150 and 15 µg disk-1. The fraction K.J.16.U.1.4.4 exhibited stronger activity than that exhibited by chloramphenicol at 150 µg disk-1. The sponge *Reniera* sp. collected from Karimunjawa Islands comprise bacterial and fungal isolates produced antibacterial compounds that inhibited the growth the MDR *E. coli* and *S. aureus* strains.

Keywords: Associate microorganisms, bioactive compounds, MDR pathogen, sponges *Reniera*

INTRODUCTION

The sponge is a primitive marine organism belongs to phylum Porifera that has important ecological roles as substrate stabilizer, bio-eroding, a shelter for small organisms, and in the cycle of silicon, carbon, nitrogen, and other nutrients (Bell 2008). As a sessile and filter feeder organism, the sponge is susceptible to physical damage, predatory attacks, and pathogenic infections. Sponge have developed the specific morphology, physiology, ability to produce diverse bioactive metabolites to support their survival. Some of the secondary metabolites possessed interesting bioactivities such as antibacterial (Salim et al. 2012), antifungal (Taylor et al. 2007), antiviral, anti-inflammatory, antinociceptive (Souza et al. 2009), anticancer, and antioxidant (Trianto et al. 2011).

The *Reniera* sp. one of the sponges belongs to the Chalinidae family that well known as a rich source of the bioactive compounds, have attracted many researchers. Saito et al. (2004) isolated several bioactive compounds belong to reniera mycin, mimosamycin, and renierone that have bioactivity as antiproliferative against several cancer cells. Lunder et al. (2012) published the polymeric 3-alkylpyridinum salts, isolated from the sponge *Reniera sarai*, a unique water-soluble compound that has a cardiotoxic effect. Urda et al. (2018) exhibited the bioactive compounds from Reniera sponge called Njoamines, a member of the alkaloid family contains a tri-cyclic nitrogenated core.

The surface or the inner parts of the sponges rich in nutrients than those present in seawater and sediments that suitable habitats for microorganisms. Some of these microorganisms are used as food while others live in symbiosis with the sponge as their host. Although the interaction between sponges and microorganisms is not yet clearly understood, there is an exchange of nutrients and secondary metabolites, which suggests that the survival of the sponge presumably depends on the positive host-symbiont relationship. This kind of interaction may contribute to the nutrient acquisition and production of secondary metabolites in responsibility to protect the sponge body from microbial infection (Engel et al. 2002). Some of the bacteria living symbiotically with the sponge produce bioactive compounds (Kikuchi et al. 2018). Marine sponges are also rich in fungal symbionts that produce antibacterial and fungal compounds (Trianto et al. 2017a, b,). Marine microbes, both living freely and in association with the sponges, have been known to produce various bioactive compounds. The purpose of this work is to assess antibacterial the potential of bacteria and fungi isolated from sponge *Reniera* sp. collected from

Karimunjawa Islands, Indonesia against two multi-resistant resistance bacteria (MDR).

MATERIALS AND METHODS

Sponge collection and isolation of sponge microbial symbionts

The sponge *Reniera* sp. was collected from Karimunjawa Islands, Jepara, Central Java, Indonesia, from the depth of 2-5 m by skin diving method. Each sponge specimen was put into a sterile zip-lock plastic bag and stored in a cool box until the isolation process. The specimens were washed with the sterile seawater prior to microbial isolation. The fungal isolates were isolated from the sponge by tapping method. A small part (ca.1 cm x 1 cm x 0.3 mm) of was cut from each specimen, and the sponge part was put onto the malt extract agar (MEA) media surface. While the microbial symbionts were isolated using the serial dilution method. The tissue solutions were spread on Zobell marine agar plates (Radjasa et al. 2007). Identification of the sponge was based on the in situ picture and spicule analysis (Hooper 2003; Kang et al. 2013).

Screening for antibacterial activity

Both of the bacterial and the fungal isolates were screened for the antibacterial activity against the pathogenic bacteri MRSA and MDR *E. coli* using the modified overlay method described by Anand et al. (2006). The bacterial symbionts were spotted on Zobell marine agar medium and incubated for three days, while the fungal strains were inoculated in the MEA medium and incubated for four days. The fresh culture of the pathogens was mixed with soft agar medium (1% v/v) and overlaid over the agar surface of the previous microbial symbiont cultures. The plates were then incubated at 37°C for 24 h. The antibacterial activity was defined by the presence of clear zones around the microbial symbiont.

Gram test

The bacterial Gram identification was carried out using the KOH 3% string and Gram staining tests. KOH 3% string test was done according to Ali et al. (2015); Dash and Payyappilli (2016), a loopful of the bacterial colony from the culture plate was emulsified over glass slide in the suspension of 3% KOH. Gram staining test was done according to Ayuningrum et al. (2017), using Gram's staining solution A (crystal violet), solution B (Lugol iodine), solution C (alcohol) and solution D (safranin).

Mass culture and extraction of microbial symbionts

The isolates with the strongest activity were chosen for further studies. The most active isolates, a bacterium, and three fungal isolates were cultured for the production of the bioactive compounds. The bacterial inoculum was prepared in 5-mL Zobell broth medium on a rotary shaker at room temperature, after 24 h, the inoculum was serially transferred into larger volumes (10, 100, and 1000 mL). The fungi were cultured in malt extract broth (MEB) medium at room temperature for several days (Radjasa & Sabdono 2003). The microbial mass was separated from the culture medium for extraction of the bioactive substances. The bacterial culture was centrifuged at 1000 rpm for 10 min, the supernatant was discarded, and the pellet was soaked in MeOH for 24 h. The fungal mycelia were separated from the culture medium by filtration with a paper filter; then the mycelia were soaked in MeOH for 24 h. The solution was filtered and concentrated with the rotary evaporator in vacuo to obtain the crude extracts. The extracts were partitioned using a solvent mixture of ethyl acetate and H_2O solvents to provide the organic and water fractions.

Antibacterial test of the extract

The antibacterial test was conducted by the disk diffusion method. The extracts were dissolved in ethyl acetate to prepare the stock solutions with the concentration 1.5 µg µL-1, then 10 µL of the stock solutions was impregnated into a sterile paper disk with diameter 5 mm in to give the final concentration at 15 µg disk-1. The paper disks were placed on the nutrient agar medium surface that was previously inoculated with the pathogenic bacteria. The standard antibiotic (chloramphenicol, 15 µg disk-1) was used in this test. The antibacterial activity was defined based on the inhibition zones around the paper disk (Ozdemir et al. 2006).

Molecular identification

The DNA extraction was carried out using the Chelex method (Walsh et al. 1991). Selected colonies were inoculated in 50-100 µL ddH2O and 1 mL of 0.5% saponin in phosphate buffer solution (PBS) 1× (stored overnight). The mixture was centrifuged at 2000 rpm for 10 min (1 rpm = 1/60 Hz), and then the supernatant was discarded. Then, 100 µL ddH2O and 50 µL of 20% Chelex 100 were added to the final solution, and the solution was boiled for 10 min and vortexed once for 5 min. The mixture was centrifuged at 12000 rpm for 10 min and stored at-20°C.

The bacterial DNA that was used for 16S rRNA gene sequencing was amplified by polymerase chain reaction (PCR) using the universal primers 27F (5′AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′TACGGTTAACCTTG TTACGACTT-3′). The fungal DNA that was used for 5.8S rDNA gene sequencing was amplified by PCR with the internal transcribed spacer (ITS) region ITS 1 (5′TCCGTAGGTGAA CCTGCGG-3′) and the primer ITS4 (5′TCCTCCGCTTATTGATATGC-3′).

The DNA sequencing was conducted at Macrogen Laboratory, Korea. The results of DNA sequences were preliminarily aligned with ClustalW Multiple Sequence Alignment. Phylogenetic analysis was performed using MEGA 6. The phylogenetic trees were determined by the neighbor-joining method using Kimura's two-parameter model.

RESULTS AND DISCUSSION

Isolation the microbial and screening for antibacterial activity of the isolates

A total of 46 bacteria and 43 fungi were isolated from the sponge *Reniera* sp. Among the isolates, 5 bacteria inhibited *S. aureus* and *E. coli,* and 3 of the active isolates inhibited both pathogenic bacteria. Whilst, 19 and 7 fungal isolates inhibited *S. aureus* and *E. coli,* respectively. Among the active fungal isolates, 6 of them inhibited both bacteria (Figure 1). The preliminary test revealed that 23.6% of the strains were capable of inhibiting the MDR pathogens MRSA and *E. coli*. The antibacterial test of the isolates extract showed one bacterium along with three fungal isolates having strong activity against the antagonist bacteria.

The *Reniera* sponge is known to have symbiotic microbes either bacteria or fungi. Sponge *Reniera* sp. collected from Panjang Island, Jepara Central Java contains five associated bacteria that one of the bacteria is *Chromobacter salexigens* strain DSM 3043 active against *P. aeruginosa, E. cloaceae, A. baumannii,* and *S. aureus* (Asagabaldan 2017). The microbial community plays important roles in sponge survival and adaptation to environmental change. The microorganisms serve the food supply of the sponge both internal exchange and with the environment (Kiran et al. 2018). Bacteria also produced bioactive compounds that protect the sponge from biofouling organisms (Aguila-Ramírez et al. 2014; Satheesh et al. 2016).

Bioactivity of bacterial biomass and fungal mycelia extract

The most active isolates were cultured in higher mass to provide the extract for further study. The extraction of the bacterial biomass and fungal mycelia yielded methanolic extract with concentrations of 5.3-18.7%. The methanol extraction yielded crude extract that was partitioned with H_2O and ethyl acetate before conducting the antibacterial assay. The antibacterial screening results are shown in Table 1.

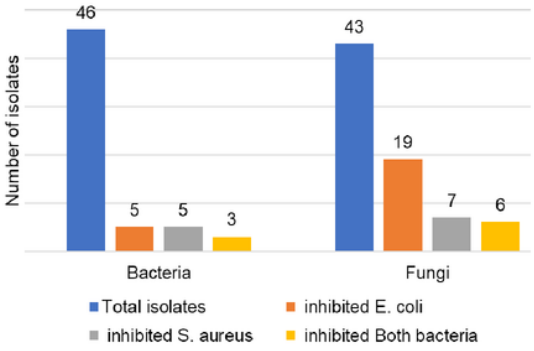

Figure 1. The number of total and active bacterial and fungal isolates of the sponge *Reniera* sp.

Table 1. Bioactivity of ethyl acetate extract of the bacterial biomass and fungal mycelia obtained from 1liter batch culture

Isolat	Bacterial/ Mycelial weight (g)	Extract weight (mg)	Extract concentration (%)	The diameter of the inhibition zone (mm)	
				E. coli	MRSA
SKSBT.0.3.29	6.6	348.4	5.3	2.3	7.2
SKSJT. J. 16 H	17.9	3340.0	18.7	10.3	10.6
SKSJT. J. 16 U	31.0	3280.0	10.6	10.7	9.6
SKSJT. J.19	9.4	1300.0	13.8	7.4	2.5

Note: The diameter of the inhibition zone of (+) control chloramphenicol: 14.83 and 13.95 mm against *E. coli* and *S. aureus*, respectively. (-) control ethyl acetate and H_2O fraction did not show activity (0.00 mm).

Table 3. Identification of fungal strains isolated from *Reniera* sp. Samples were based on BLAST analysis of the internal transcribed spacer (ITS) region

Strain	Sequence length (nt)	Related cultivated strain (BLAST)	Acc. no	Similarity (%)
SKSBT.0.3.29	1165	*Pseudoalteromonas maricaloris* strain KMM636	NR 025009.1	99%
K.J.16.U	1062	*Aspergillus nomius* strain SGE19	JN709035.1	96%
K.J.16.H	545	*Eurotium rubrum* strain SGE27	JX232274.1	99%
K.J.19	983	*Penicillium* sp. DY115-21-10-M23	KF411603.1	100%

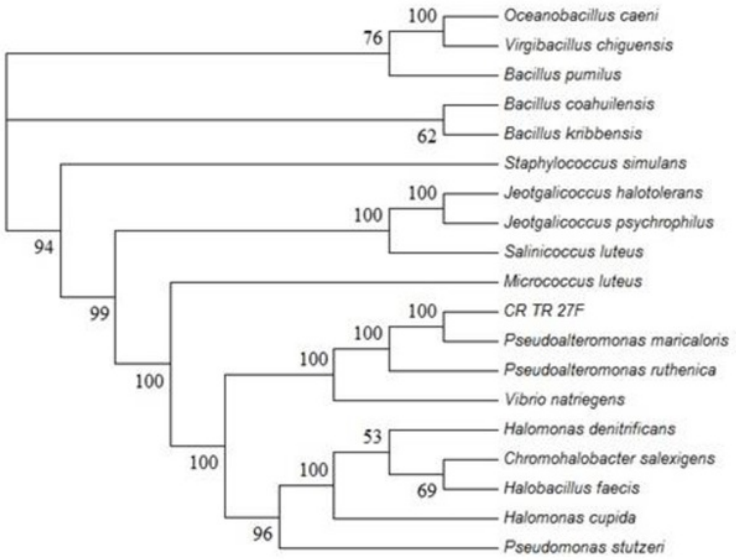

Figure 2. Phylogenetic tree of bacterial isolate

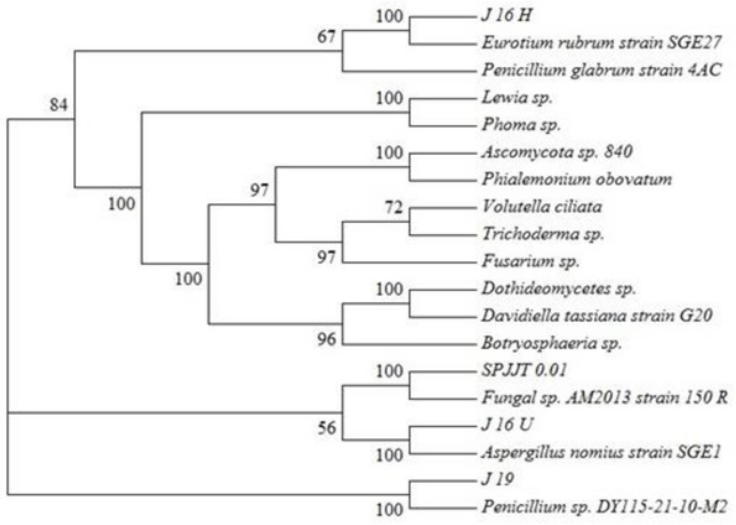

Figure 3. Phylogenetic tree of fungal isolates

Marine bacteria and fungi are well known as potential sources of natural products. For example, *Pseudoalteromonas* sp. reported producing compound pentabromopseudilin, a promising anti-MRSA and myosin ATPase inhibition with activities at a level comparable to those of commercial drug (Kum et al. 2017). Other researches showed that *Pseudoalteromonas* sp. living associated with sponge *Halisarca ectofibrosa* produce compounds that inhibit the growth of *Bacillus subtilis* and *Vibrio anguillarum* (Rungprom et al. 2008; Schinke et al. 2017). A review paper by Xu et al (2015) describes broadly the potential of marine fungi as sources bioactive compounds. The review showed there are 105 marine fungal strains used for the isolation of antibacterial or

antifungal compounds. Mangrove and sponges are the most riches sources of fungi, while *Aspergillus* and *Penicillium* are the most common fungi in the marine ecosystem. Other review depicted the high potential of marine fungi especially the genus *Aspergillus* of antibacterial and anticancer (Bladt et al. 2013).

Identification of the isolates

The ethyl acetate fraction of K.J.16.U was selected Molecular identification of the active bacterial and fungal symbionts based on 16S rRNA and 5.8S rDNA revealed that the active strains SKSBT.0.3.29, K.J.16.U, K.J.16.H, and K.J.19 were closely related to *Pseudoalteromonas maricaloris*, *Aspergillus nomius*, *Eurotium rubrum*, and *Penicillium* sp., respectively for bioassay-guided fractionation because of its high antibacterial activity, which yielded six fractions (K.J.16.U.1.4.1-6) (Table 2). The results obtained from BLAST homology of the bacterial and fungal symbionts of *Reniera* sp. are described by phylogenetic trees (Figures 2 and 3).

The molecular-based identification indicated that the active isolate is *Pseudoalteromonas maricaloris* with the BLAST homology 99% similarity. While the fungal isolates identified as *Aspergillus nomius* strain SGE19, *Eurotium rubrum* strain SGE27, and *Penicillium* sp. DY115-21-10-M23 with BLAST homology 99% similarity 96%, 99%, and 100% similarity, respectively (Table 2). Genus *Pseudoalteromonas* are heterotrophic bacteria include are essential components of the marine environment and have diverse habitats including coastal and open water areas, bottom sediments, and living associated with marine plants and animals. *P. maricaloris* has been isolated from a sponge, *Fascaplysinopsis reticulata*, collected from Australia. It has also been reported having antibacterial activity against *S. aureus*, *E. coli*, *Proteus vulgaris*, *Enterococcus faecium*, and *Bacillus subtilis* and also cytotoxic activity against tumor cells (Ivanova et al. 2002; Radjasa et al. 2007). *Pseudoalteromonas* also produces extracellular antibacterial compounds that inhibit the *Vibrio parahaemolyticus* causing acute hepatopancreatic necrosis disease (Wang et al. 2018). Asagabaldan et al. (2017) isolated bacterial strains from the sponge belonging to the genus Haliclona (Reneira) that were active against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Acinetobacter baumannii*, and *Enterobacter cloacae*.

Penicillium sp., *Aspergillus* sp., and *Eurotium* sp. have been isolated from the sponge *Tethya aurantium* collected from the Istrian Peninsula, Croatia, which possesses the secondary metabolite mycotoxin that exhibited antineoplastic properties. *A. nomius* is reported as an insect parasite and a producer aflatoxin B1, B2, G1, and G2, mycotoxins, that toxic to humans, animals, and insects (Calderari et al. 2013; Afonso et al. 2017). However, *A. nomius* has also known as lipase producer that potentially used for the production of fatty acid methyl ester in the biodiesel industry (Rakchai et al. 2016). *Eurotium rubrum* commonly found in grain products, poultry feed, bakery products, dried fruits, spices, soil, hypersaline waters, and Dead Sea soil (Butinar et al. 2005). These Eurotium reported to producing diketopiperazine compounds that potential in the cosmetic industry by inhibited melanogenesis (Kamauchi et al. 2016). The *E. rubrum* has also reported producing a metabolite called flavoglaucin, oglaucin, isotetrahydorauroglaucin, eoechinulins A and B, echinulin, preechinulin, nechinulin E, epiheveadride, and questin. The compounds have activities as anticancer, antioxidant, antibacterial, cytoprotection against peroxynitrite, hepatotoxic (Slack et al. 2009). *Penicillium* sp. is well-known for its ability to produce secondary metabolites with diverse biological effects. Penicillium sp. has been reported contains Peniopyranone, a bioactive compound inhibits acetylcholinesterase, that potential for neurodegenerative diseases such as Alzheimer's dementia and Parkinson's disease (Li et al. 2017). *Penicillium* sp. also well-known as a source of prenylated indole alkaloids having interesting biological activities such as antitumor, anthelmintic, calmodulin inhibitory, and antibacterial (Yang et al. 2018).

To conclude, the sponge *Reniera* sp. collected from Karimunjawa Islands comprise bacterial and fungal isolates produced antibacterial compounds that inhibited the growth the MDR *E. coli* and *S. aureus* strains. The fungus *A. nomius* has potential as a source of antibacterial compounds that having stronger activity against MRSA and *E. coli*.

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